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# Diverse Substrate Recognition Mechanisms for Rhomboids: Thrombomodulin Is Cleaved by Mammalian Rhomboids

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## Summary

**The rhomboids are a recently discovered family of intramembrane proteases that are conserved across evolution. *Drosophila* was the first organism in which they were characterized, where at least Rhomboids 1–3 activate EGF receptor signaling by releasing the active forms of EGF-like growth factors [1, 2]. Subsequent work has begun to shed light on the role of these proteases in bacteria and yeast [3–8], but nothing is known about the function of rhomboids in vertebrates beyond evidence that the subclass of mitochondrial rhomboids is conserved [4]. Here, we report that the anticoagulant cell-surface protein thrombomodulin [9] is the first mammalian protein to be a rhomboid substrate in a cell culture assay. The thrombomodulin transmembrane domain (TMD) is cleaved only by vertebrate RHBDL2-like rhomboids. Thrombomodulin TMD cleavage is directed not by sequences within the TMD, as is the case with Spitz but by its cytoplasmic domain, which, at least in some contexts, is necessary and sufficient to determine cleavage by RHBDL2. These data suggest that thrombomodulin could be a physiological substrate for rhomboid. Moreover, the discovery of a second mode of substrate recognition by rhomboids implies mechanistic diversity in this family of intramembrane proteases.**

## Results and Discussion

### Identifying Mammalian Rhomboid Substrates

Based on the hypothesis that rhomboids in *Drosophila* and mammals might have a conserved role in EGFR signaling [1, 10], we initially investigated whether mammalian membrane-tethered EGFR ligands were cleaved by human RHBDL1 and RHBDL2; of the seven tested, none were cleaved by either human rhomboid (see Supplemental Data).

We next searched the mouse genome sequence for single transmembrane domain proteins with the widely conserved rhomboid substrate motif, previously characterized in the *Drosophila* ligand Spitz [4, 11]. This substrate motif depends on predicted protein conformation rather than primary sequence, making the search somewhat subjective and difficult to automate. A manual search through about 50% of genes in the mouse genome annotated as having a TMD and signal peptide (approximately 1200 searched) revealed that only 12 appeared to be good candidates for Spitz-like substrates (Table S1). One of these was the anticoagulant protein thrombomodulin, which comprises a large N-terminal domain with homology to lectins and six EGF repeats; a TMD; and a highly conserved, short cytoplasmic domain with no recognizable motifs [9, 12].

The thrombomodulin TMD resembles the Spitz TMD in a number of significant respects (Figure 1A), so we tested whether human thrombomodulin could indeed be cleaved by human RHBDL1 or RHBDL2 [1, 13] when the proteins were coexpressed in mammalian cells using a previously described assay [1]. First, C-terminally tagged thrombomodulin was assayed, and a cleaved band of expected size was detected in lysates from cells coexpressing RHBDL2, but not RHBDL1 (Figure 1B). The experiment was repeated with N-terminally tagged thrombomodulin and the extracellular domain accumulated in the medium, again in response to RHBDL2 only (Figure 1C). Finally, an antibody against the extracellular domain of thrombomodulin was used to detect the accumulation of untagged extracellular domains in the medium triggered by specifically RHBDL2 (Figure 1D). As with other rhomboid substrates [1], thrombomodulin cleavage was insensitive to the broad-spectrum metalloprotease inhibitor batimastat and was abolished when the putative catalytic serine of rhomboid was mutated to alanine (Figures 1B–1D).

Thrombomodulin was cleaved at significantly lower levels than cleavage of the *Drosophila* substrate Spitz (Figure 1E). To address whether this might indicate that thrombomodulin proteolysis was a nonspecific artifact caused by overexpression of RHBDL2, we reduced the levels of RHBDL2 expression over a range of $10^3$-fold by reducing the amount of specific DNA in each transfection. 100-fold reduction of input rhomboid DNA reduced the expression of RHBDL2 to undetectable levels, but thrombomodulin cleavage was barely affected; and even when input DNA was reduced 1000-fold, cleavage was still detectable (Figures 1E and 1F). This substoichiometric requirement resembled the cleavage of Spitz by *Drosophila* Rhomboid-1 [1].

These results confirm that at least in our cell culture assay, human thrombomodulin is cleaved by RHBDL2, but not RHBDL1. We tested whether RHBDL2 was specific for thrombomodulin by assaying whether another single TMD protein in the same functional clotting complex as thrombomodulin, the endothelial protein C receptor (EPCR) [14], was cleaved. Under the same conditions, no EPCR cleavage was detected (Figure 1G). We also assayed RHBDL2 cleavage of Pref-1 (also known as Dlk1), a mouse protein with structural similarity to thrombomodulin [15], which was in our list of 12 candidate substrates; again, no cleavage was detected (not shown). Moreover, the TMDs of the other 10 proteins that we identified as potential rhomboid substrates were also uncleaved by mouse RHBDL2 when expressed as

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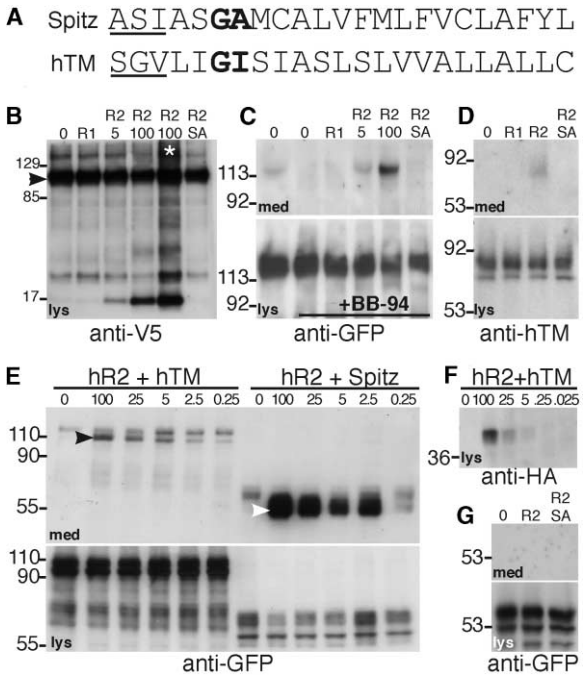


Figure 1. Human Thrombomodulin Is Cleaved by RHBDL2

(A) Alignment of the human thrombomodulin and *Drosophila* Spitz transmembrane domains. The conformationally similar residues are highlighted: small, relatively hydrophilic, and β-branched residues in the top three positions (underlined) followed by a potentially helix-destabilizing pair of residues (bold).

(B–E) Western blots of lysates (lys) or media (med) from COS or NIH3T3 cells transfected with combinations of human thrombomodulin and rhomboids. (B) C-terminally V5-tagged thrombomodulin was coexpressed in NIH3T3 cells with human RHBDL1 (R1), 5 or 100 ng of RHBDL2 (R2), or 100 ng of an active site mutant of RHBDL2 (R2-SA); 250 ng of thrombomodulin-V5 DNA was used in each lane except that marked by a white asterisk, in which 750 ng was used. Full-length thrombomodulin is indicated (arrowhead); the tagged cleaved form runs adjacent to the 17 kDa marker. (C) N-terminally GFP-tagged thrombomodulin was coexpressed in NIH3T3 cells with RHBDL1 (R1), 5 or 100 ng of RHBDL2 (R2), or 100 ng of an active site mutant of RHBDL2 (R2-SA). The metalloprotease inhibitor batimastat (BB-94; 20 μM) was included in all but the first lane to inhibit background shedding of cell surface proteins. (D) Thrombomodulin was similarly coexpressed in NIH3T3 cells with rhomboids, and anti-thrombomodulin extracellular domain (Santa Cruz) was used to confirm RHBDL2-specific shedding; this blot was done under nonreducing conditions. In this and subsequent experiments, 20 μM batimastat was included in all assays unless otherwise noted. (E) Titration of RHBDL2 activity against thrombomodulin (left lanes) and Spitz (right lanes) in COS cells. In each case, decreasing levels of RHBDL2 DNA was included in the transfection (shown in ng; normally 250 ng per transfection is used). The GFP-tagged shed products were detected in medium; thrombomodulin and Spitz indicated by black and white arrowheads, respectively. Expression of the substrate was monitored in cell lysates (lys).

(F) Under the same conditions, the expression of HA-tagged RHBDL2 was followed; unlike the cleavage of substrates, RHBDL2 expression decreased proportionally to input DNA.

(G) N-terminally GFP-tagged endothelial protein C receptor (EPCR) was not shed in NIH3T3 cells in response to RHBDL2.

part of chimeric molecules analagous to the "TM" chimera in Figure 4 (not shown). This suggests that our ability to predict substrate TMDs from sequence alone is limited. More positively, taken with the other TMD

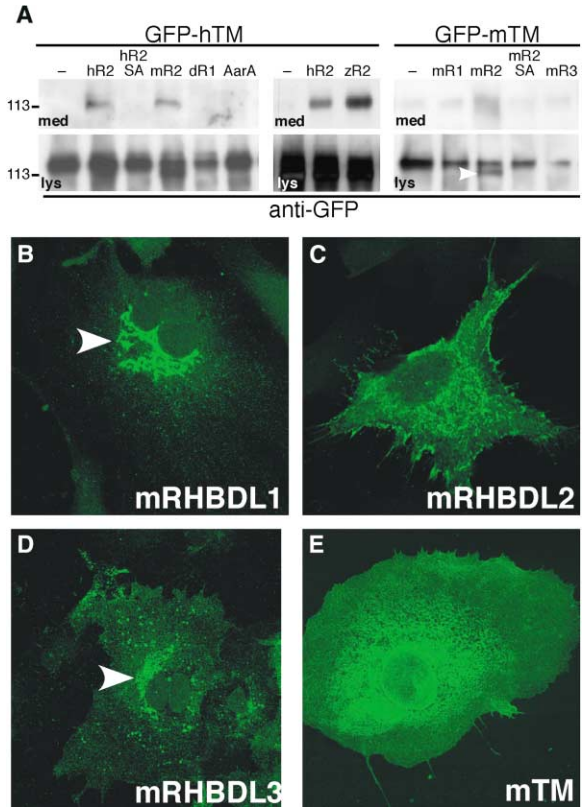


Figure 2. Specificity of Thrombomodulin Cleavage

(A) A variety of rhomboids were cotransfected with GFP-tagged human thrombomodulin (left two panels) or mouse thrombomodulin (right), and the thrombomodulin released into the medium was detected by Western blot. Left: human thrombomodulin was cleaved in NIH3T3 cells by human (hR2) and mouse RHBDL2 (mR2), but not by *Drosophila* Rhomboid-1 (dR1) or AarA from *Providencia stuartii*. Middle: human thrombomodulin was also cleaved in COS cells by the apparent RHBDL2 ortholog from the zebrafish *Danio rerio* (zR2). Right: mouse thrombomodulin was cleaved in NIH3T3 cells only by mouse RHBDL2; this is most apparent in the lysate, where the cleaved but not-yet-secreted band is indicated by an arrowhead. Note that there is a background band in every lane of the medium blot; the specific cleaved product is slightly smaller.

(B–E) Immunofluorescent staining of COS cells transfected with HA-tagged mouse rhomboids and GFP-tagged mouse thrombomodulin is shown. In (B), RHBDL1; in (C), RHBDL2; in (D), RHBDL3; and in (E), thrombomodulin.

proteins that are uncleaved by rhomboids [3, 11], these results further indicate the specificity of TMD cleavage by rhomboids, suggesting the possibility that thrombomodulin cleavage might have physiological significance.

## Only RHBDL2-like Rhomboids Cleave Thrombomodulin

The ability of RHBDL2, but not RHBDL1, to cleave thrombomodulin led us to examine whether other rhomboid proteases could also cleave thrombomodulin, by analogy to the cleavage of *Drosophila* Spitz by many rhomboids. Human thrombomodulin was cleaved by human and mouse RHBDL2 and the zebrafish ortholog of RHBDL2; however, it was not cleaved by *Drosophila* Rhomboid-1 or the bacterial rhomboid AarA, both of which cleave Spitz [1, 3] (Figure 2A). We also tested the

cleavage of mouse thrombomodulin by all of the three nonmitochondrial mouse rhomboids identifiable in the mouse genome; only RHBDL2 showed activity (Figure 2A). In all cases where cleavage occurred, it was dependent on the presence of the catalytic serine of RHBDL-2: activity was abolished when the serine was mutated to alanine (not all shown). Note that the specificity implied by our conclusion that only RHBDL2 cleaves thrombomodulin must be qualified by the fact that no substrate has yet been found for RHBDL1 and RHBDL3 (also known as ventrhoid [16]) (our unpublished data). Both contain all the conserved catalytic residues, so we presume they are active proteases, but it remains possible that they lack proteolytic activity.

A potential explanation for the inability of RHBDL1 and RHBDL3 to cleave thrombomodulin would be that the enzymes and substrates are segregated from each other within the cell. To address this, we examined the localization of HA-tagged mouse RHBDL1, 2, and 3 using protocols previously described [10]. All three were localized in the secretory pathway, specifically the Golgi apparatus and the plasma membrane. Although there was some variability, RHBDL1 was quite restricted to the Golgi apparatus (Figure 2B, arrow) and only weakly detected at the plasma membrane; conversely, RHBDL2 was predominantly at the plasma membrane (Figure 2C); RHBDL3 was seen weakly in the Golgi apparatus (Figure 2D, arrow) but also at the plasma membrane and in dots that appear to be endosomes. GFP-tagged thrombomodulin was also located in the secretory pathway–visible in the ER, the Golgi apparatus, and the plasma membrane (Figure 2E). These data indicate that differential compartmentalization cannot account for the specificity of RHBDL2 for thrombomodulin.

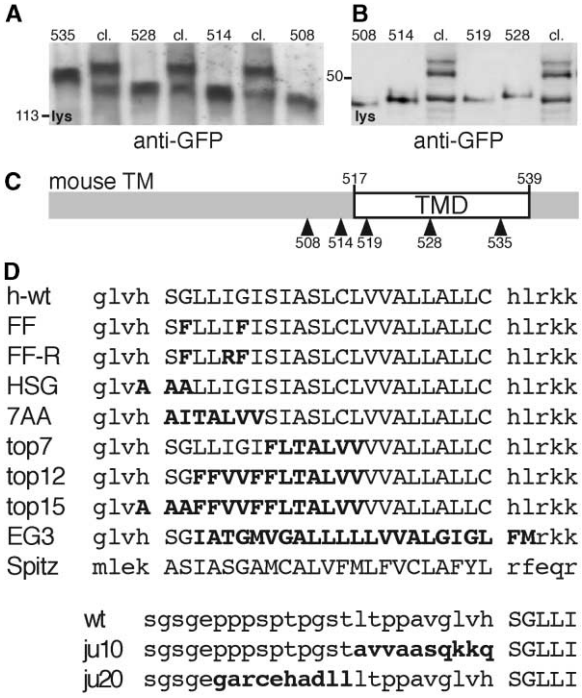


Figure 3. Thrombomodulin Is Cleaved in Its TMD

(A) The RHBDL2 cleavage site of mouse thrombomodulin was mapped by comparing in cell lysates the cleaved product (lower band in lanes marked "cl.") with truncations of thrombomodulin that precisely mimic cleavage at the indicated amino acid residues. The cleaved product was perceptibly smaller than the 528 truncation but larger than the 508.

(B) A similar analysis was performed with thrombomodulin with a large N-terminal deletion. Coordinates correspond to the same numbering system as in (A).

(C) A diagram of the position of the coordinates used in the mapping.

(D) A series of TMD (top group) and juxtamembrane (bottom group) mutants of human thrombomodulin were assayed for their ability to be cleaved by RHBDL2. Mutations are shown in bold; none abrogated cleavage.

## RHBDL2 Cleaves Thrombomodulin near the Top of Its TMD

Rhomboids are unique among proteases responsible for shedding eukaryotic extracellular signaling domains in that instead of cleaving in the extracellular juxtamembrane region, they are intramembrane proteases that cut within the TMD [1]. There was insufficient cleaved extracellular domain of thrombomodulin in medium to allow a direct biochemical determination of the RHBDL2 cleavage site. We therefore mapped it by comparing the size of the cleaved, GFP-tagged N-terminal fragment with identically tagged artificial truncations of the protein. In the first series of experiments, the cleaved fragment of full-length mouse thrombomodulin in cell extracts was compared with C-terminal truncations that contained the whole N-terminal region. The fragment was larger than truncations at residue 508 but smaller than truncations at residue 528 (Figure 3A). This located the approximate site of cleavage to between residues 510 and 525 (note that we predict the TMD to run from residues 517–539).

We performed a second set of experiments in which most of the thrombomodulin N terminus had been removed, resulting in smaller protein fragments and more precise size comparisons. This series gave results consistent with the first series, and the cleaved fragment was larger than 514 but indistinguishable from the 519 truncation (Figure 3B). We conclude that the site of thrombomodulin cleavage is most likely at residues 518, 519, or 520, corresponding to the top region of the TMD (Figure 3C). Importantly, this result strongly supports the conclusion that thrombomodulin is a direct substrate of RHBDL2, as rhomboids are the only proteases known to cleave within TMDs near the luminal/extracellular side.

Spitz-like rhomboid substrates depend on helix-destabilizing residues in the top part of their TMDs and require reasonably hydrophilic residues in the same region [11]. Based on this requirement and our previous ability to abrogate cleavage with TMD mutations, we made an extensive set of mutations in the TMD and the juxtamembrane region of thrombomodulin (Figure 3D), but none of these changes prevented or substantially reduced cleavage by RHBDL2 (not shown). This inability to block RHBDL2 cleavage suggests that it might occur by a distinct mechanism to the cleavage of Spitz. This would be consistent with our observation that bacterial and *Drosophila* rhomboids cannot cleave thrombomodulin, whereas they cleave Spitz efficiently. There is already evidence that Spitz-type recognition is not the only mechanism that exists: the *Drosophila* EGFR ligand

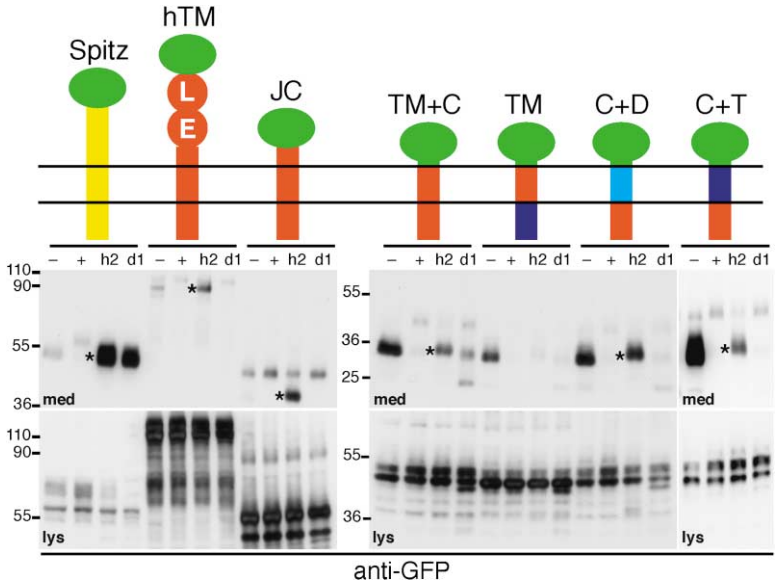


Figure 4. The Cytoplasmic Domain of Thrombomodulin Directs RHBDL2 Cleavage

A panel of GFP-tagged potential RHBDL2 substrates was assayed by Western blot by coexpressing them in COS cells with human RHBDL2 (h2) or *Drosophila* Rhomboid-1 (d1); in each set of four lanes, the left-hand lane is a shedding control without batimastat, while all other lanes show assays performed in the presence of 20 μM batimastat. The bands corresponding to RHBDL2 shedding are indicated by asterisks. The substrate is indicated diagrammatically above each set of four lanes; see Experimental Procedures for coordinates of chimeras. Color code: green, GFP; yellow, Spitz; red, thrombomodulin (which, when full-length, includes lectin (L) and EGF (E) motifs); dark blue, TGFα; light blue, *Drosophila* Delta.

Gurken is efficiently cleaved by rhomboids but does not appear to conform to the structural constraints identified in Spitz [2, 11].

### The Cytoplasmic Domain of Thrombomodulin Directs Its Cleavage by RHBDL2

Since the TMD mutations did not prevent thrombomodulin cleavage, a series of domain swaps and deletions were used to map the determinants that allow it to be cleaved by RHBDL2 (Figure 4). Removal of either the N-terminal portion (JC chimera) or the whole (TM+C chimera) of the extracellular domain did not abrogate cleavage. However, a chimera comprising an extracellular tag, the thrombomodulin TMD, and a cytoplasmic domain from TGFα (TM chimera), was not cleaved. This implied that unlike Spitz, the TMD of thrombomodulin is not sufficient to confer cleavage/recognition by RHBDL2. Furthermore, it demonstrated that again, unlike Spitz, the cytoplasmic C terminus of the protein is necessary. To examine the role of the cytoplasmic domain of thrombomodulin further, we tested whether it was also sufficient for RHBDL2 cleavage. Strikingly, the cytoplasmic domain of thrombomodulin was sufficient to transform the TMD of either *Drosophila* Delta or human TGFα—both type 1 transmembrane proteins—into RHBDL2 substrates (Figure 4, chimeras C+D, C+T). These experiments imply that the cytoplasmic domain of thrombomodulin is both necessary and sufficient for the cleavage of the thrombomodulin TMD. It is also sufficient to direct cleavage by RHBDL2 of at least two other TMDs that are not otherwise substrates.

One interpretation of these data is that the cytoplasmic domains of RHBDL2 and thrombomodulin participate in the enzyme/substrate recognition mechanism. We have begun to investigate this by deleting the N terminus of RHBDL2. This significantly reduced its activity against thrombomodulin (Figure 5A), suggesting a function for the RHBDL2 cytoplasmic domain. However, substituting the N-terminal cytoplasmic domain of *Drosophila* Rhomboid-1 with the equivalent domain of RHBDL2 was not sufficient to transform Rhomboid-1 into an enzyme that could cleave thrombomodulin, although the chimeric rhomboid retained activity against Spitz (Figure 5B). These results support the idea that the cytoplasmic domains of thrombomodulin and RHBDL2 are involved in the recognition of the substrate, although they also suggest that other parts of RHBDL2 participate.

### Function of Thrombomodulin

The role of thrombomodulin in the protein C anticoagulation pathway is well established [reviewed in 9, 12]. It is expressed on endothelial cells that line the blood vessels where it forms a complex with the clotting factor thrombin, inhibiting thrombin's interaction with fibrinogen. At the same time, the thrombin-thrombomodulin

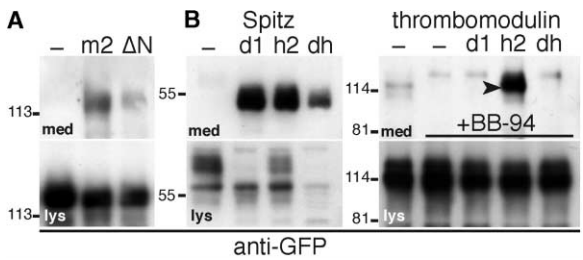


Figure 5. The N-Terminal Cytoplasmic Domain of RHBDL2 Participates in Thrombomodulin Cleavage

(A) Thrombomodulin shedding into the medium (med) by mouse RHBDL2 (m2) is reduced when its cytoplasmic domain is deleted (ΔN); the lysate (lys) panel shows that thrombomodulin was expressed at equivalent levels. This experiment was done in the presence of 20 μM batimastat.

(B) Left: *Drosophila* Rhomboid-1 (d1), human RHBDL2 (h2), and a chimera comprising *Drosophila* Rhomboid-1 with its N terminus replaced by the equivalent domain of human RHBDL2 (dh) all shed Spitz. All assays done in presence of 20 μM batimastat. Right: in contrast, thrombomodulin is shed only by RHBDL2 (arrowhead), but not by *Drosophila* Rhomboid-1 or the chimera. In this case, batimastat (BB-94) was added to the assays shown in all but the left-most lane. Coordinates of all constructs are given in the Experimental Procedures.

complex activates protein C, which proteolyses the activated coagulation factors Va and VIIIa. These two activities give thrombomodulin an important anticoagulant role. Beyond this, the biology of thrombomodulin is less well understood although it has been implicated in many processes including inflammation, adhesion, tumorigenesis, and embryonic development (reviewed in [9]).

A circulating form of thrombomodulin, shed from the cell surface, is normally present in plasma and other fluids [17], implying that it is cleaved under physiological conditions. But it is not known whether soluble thrombomodulin has a function or whether it is merely a marker of endothelial cell damage. Circulating products representing a variety of cleavage sites can be found in plasma [17]. Most correspond to proteolysis in the region between the membrane and the EGF repeats, but some are large enough potentially to correspond to intramembrane cleavage. Little is known about the proteases responsible for thrombomodulin shedding, although neutrophil-derived enzymes including elastase, proteinase-3, and cathepsin G have been implicated [17, 18].

## Conclusions and Perspectives

Our discovery that thrombomodulin is efficiently and specifically cleaved by RHBDL2, coupled with our observations reported here and previously that most TMDs are not rhomboid substrates, suggests that this cleavage may be physiologically significant. If so, this would be the first vertebrate rhomboid substrate to be discovered and would represent a new biological function for the rhomboid family of proteases, which are conserved throughout evolution [19–21].

Beyond the obvious significance of a potential role for RHBDL2 in thrombomodulin release, our work has implications for studying rhomboids. One of the most efficient methods for probing rhomboid function is to identify substrates: these provide insight into the cellular processes that rhomboids mediate. The knowledge that a second type of substrate recognition mechanism can be used by some rhomboids might influence strategies for finding rhomboid substrates. Finally, the discovery of a second mode of substrate recognition by rhomboids implies mechanistic diversity in this family of intramembrane proteases. Note, however, that the two recognition mechanisms we have uncovered are not mutually exclusive: as well as cleaving thrombomodulin, human, mouse, and zebrafish, RHBDL2s can also cleave Spitz [11] and do so by recognizing the standard Spitz TMD motifs, implying that these are enzymes with dual specificities.

### Supplemental Data

Supplemental Data including Experimental Procedures, a description of the mammalian EGFR ligands tested for cleavage by rhomboids, and a list of the 12 mouse proteins selected as possible rhomboid substrates are available online at http://www.current-biology.com/cgi/content/full/14/3/236/DC1/.

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